

Effects of double stress on antioxidant enzyme activity in *Vigna radiata* (L.) Wilczek

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Abstract – The effects of double stress environment i.e. lead (heavy metal) and NaCl (saline) on the activity of antioxidant enzymes in *Vigna radiata* seedling were studied. The antioxidant activities of enzymes, i.e. of superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase, glutathione reductase and their activity proportions were examined. Superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase and glutathione reductase activities were substantially increased in a combined stress environment as compared to catalase. Further, in comparison with catalase and ascorbate peroxidase, glutathione reductase showed increased activities together with superoxide dismutase in a combined stress environment. Superoxide dismutase and glutathione reductase showed higher activity proportion in combined treatment. Physiological role of these enzymes in stress tolerance mechanism is discussed.

KeyWord: Antioxidant, catalase, enzyme activity, glutathione reductase, guaiacol peroxidase, ascorbate peroxidase, seedling, stress, superoxide dismutase, *Vigna radiata*

Abbreviations: APX – ascorbate peroxidase, CAT – catalase, GPX – guaiacol peroxidase, GR – glutathione reductase, ROS – reactive oxygen species, SOD – superoxide dismutase

Introduction

Environmental stress factors like drought, temperature, high salinity and heavy metals are the major constraint that limit plant growth and productivity, by disturbing the intracellular water balance. Usually, in fields or on agricultural land, unlike in a laboratory or even a greenhouse environment, plants are subjected to a manifold array of stress factor (SIDDIQUI et al. 2008, NAWAZ et al. 2010). However, most of the studies have been devoted to assess the physiological response of plants in a single stress environment like salinity (SINHA 1991, SHARMA and GILLS 1994, KUMAR and KUMAR 1996), drought (SHINOZAKI and YAMAGUCHI 2007) and heavy metals (HAMEED et al. 2000, JETLEY et al. 2004) and studies on the physiological responses of plants under a combination of such stresses are restricted to

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just a few reports (WANG et al. 2003, DUDLEY and SHANI 2003, WANG and HUANG 2004, JAKAB et al. 2005) and even they are not directly related to the combination of stress factors like heavy metal and salt stress.

Plants have utilized various mechanisms to combat with abiotic stresses. Among them, stress tolerance gene expression, compatible solutes, phenols and antioxidant enzyme production are some examples (JAKAB et al. 2005, AHMED et al. 2010, SIDDQUI and KHAN 2011). Antioxidant enzymes such as superoxide dismutase (SOD) ascorbate peroxidase (APX), glutathione reductase, guaiacol peroxidase and catalase are well-known defense systems providing protection against the hazards of reactive oxygen species (ROS) in different stressful conditions (ALLEN et al. 1997, KWON et al. 2002, AHMED et al. 2010). Activities of these antioxidant enzymes are frequently observed in a single stress environment but the response and the proportion of the relative activities of these enzymes in a combined heavy metal (lead) and salt stress environment have seldom been reported. To make up for this lack, the present study examines the response of antioxidant enzymes and the proportions of their relative activities in a combined heavy metal (lead chloride and lead nitrate) and salt (NaCl) stress environment. Lead is used in two forms to differentiate the effects of lead as chloride or nitrate together with sodium chloride environments.

Material and methods

Germplasm of *Vigna radiata* was obtained from the Pakistan Agriculture Research Centre Karachi University. Seeds were sterilized with 50% Clorox for 10 min, and then repeatedly washed with distilled water. The sterilized seeds were placed on moist Whatman 1 filter paper in Petri dishes and kept at 25 °C in the dark until germination. Germinating seeds were transferred into small plastic pots in a plastic tray (diameter 10cm, height 12.5 cm) containing acid-washed sand. The whole set up was kept in a growth chamber with a 13-h photoperiod (28 °C, $600 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) and 11 h night (22 °C) at 60–70% humidity. Plants were irrigated with 3 L of half-strength Hoagland's solution (HOAGLAND et al. ARNON 1950) until the plant developed five to six expanded leaves. Treatments were prepared in this order (T1= 15 and 30 mg L⁻¹ lead chloride and lead nitrate, T2 = 75, 150 and 225 mM NaCl, T3 = 15 mg L⁻¹ and 3 mg L⁻¹ lead chloride and nitrate in 75, 150 and 225 mM NaCl, T4 = half strength Hoagland solution (control). All the treatments were prepared in half strength Hoagland solution and poured separately in large size (30.5 cm) plastic plates in which pots were kept on alternate days. Plants were allowed to grow up to three weeks. Leaf samples of three weeks old plants were randomly collected from the treatments and the control and subjected to antioxidant enzyme activity estimation. All the treatments and controls were replicated four times.

Extraction

Randomly collected 500 mg leaf samples were crushed in liquid nitrogen at 4 °C and homogenized in 10 mL protein extraction buffer containing Tris-HCl pH 6.8, 50 mg PVP, 10 mL DDT, 0.1 mM EDTA. The contents were centrifuged at 10,000 RPM for 15 min. Total protein was estimated by the method of BRADFORD (1976)

Catalase (Enzyme number by NC IUBMB: EC 1.11.1.6)

Catalase (CAT) activity was estimated by the method of PATTERSON et al. (1984). The decomposition of H_2O_2 was measured at 240 nm taking $\Delta\epsilon$ at 240 nm as 43.6 mM cm^{-1} . Reaction mixture (3.0 mL) consisted of 10.5 mM H_2O_2 in 0.05 M potassium phosphate buffer (pH 7.0) and the reaction was initiated after the addition of 0.1 mL enzyme extract at 25 °C. The decrease in absorbance at 240 nm was used to calculate the activity. One unit of CAT activity is defined as the amount of enzyme that catalyzes the conversion of 1 mM of H_2O_2 min^{-1} at 25 °C.

Ascorbate Peroxidase (Enzyme number by NC IUBMB: EC 1.11.1.11)

Ascorbate Peroxidase (APX) activity was determined according to the method of NAKANO and ASADA (1981). The reaction mixture (2.0 mL) contained 0.05 M potassium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid and 0.25 mM H_2O_2 . The reaction was started after the addition of 0.1 mL enzyme extract at 25 °C. The decrease in absorbance at 290 nm for one minute was recorded and the amount of ascorbate oxidized was calculated from the extinction coefficient 2.8 mM cm^{-1} . The unit of activity is expressed as micromole of ascorbic acid oxidized min^{-1} at 25 °C.

Guaiacol Peroxidase (Enzyme number by NC IUBMB: EC 1.11.17)

Guaiacol Peroxidase (GPX) activity was measured spectrophotometrically at 25 °C by the method of TATIANA et al. (1999). The reaction mixture (2.0 mL) consisted of 0.05 M potassium phosphate buffer (pH 7.0), 2 mM H_2O_2 , and 2.7 mM guaiacol. The reaction was started by the addition of 0.1 mL enzyme extract. The initial rate of guaiacol oxidation was measured by the rate of formation of tetraguaiacol and was measured at 470 nm ($\Delta\epsilon = 26.6 \text{ mM cm}^{-1}$). One unit is defined as the amount of enzyme required to catalyze the conversion of one micromole of hydrogen peroxide, with guaiacol as hydrogen donor, per minute under specified conditions.

Glutathione Reductase (Enzyme number by NC IUBMB: 1.6.4.2)

Glutathione reductase (GR) activity was determined at 25 °C by measuring the rate of NADPH oxidation as a decrease in absorbance at 340 nm ($\epsilon = 6.2 \text{ mM cm}^{-1}$) according to the method of HALLIWELL and FOYER (1978). The reaction mixture (1.0 mL) consisted of 100 mM Tris-HCl buffer (pH 7.8), 21 mM EDTA, 0.005 mM NADPH, 0.5 mM oxidized glutathione, and the enzyme extract. NADPH was added to start the reaction. Unit activity is defined by the expression »one unit will reduce 1.0 μmol of oxidized glutathione per minute under standard assay conditions«.

Superoxide Dismutase (Enzyme number by NC IUBMB: EC 1.15.1.1)

The assay for superoxide dismutase (SOD) activity was performed by following the method of BEYER and FRIDOVICH (1987). The assay mixture consisted of 27.0 mL of 0.05 M potassium phosphate buffer (pH 7.8), 1.5 mL of L-methionine (300 mg per 2.7 mL), 1.0 mL of nitroblue tetrazolium salt (14.4 mg per 10 mL), and 0.75 mL of Triton X-100. Aliquots (1.0 mL) of this mixture were delivered into small glass tubes, followed by the addition of 20 μL enzyme extract and 10 μL of riboflavin (4.4 mg per 100 mL). The cocktail was mixed and then illuminated for 15 minutes in an aluminum foil-lined box, containing 25 W fluores-

cent tubes. In a control tube the sample was replaced by 20 μL of buffer and the absorbance was measured at 560 nm. The reaction was stopped by switching off the light and placing the tubes in the dark. Increase in absorbance due to formation of formazan was measured at 560 nm. Under the described conditions, the increase in absorbance in the control was taken as 100% and the enzyme activity in the samples were calculated by determining the percentage inhibition per minute. One unit of SOD is the amount of enzyme that causes a 50% inhibition of the rate for reduction of nitroblue tetrazolium salt under the conditions of the assay.

Statistical analysis

Data were subjected to FANOVA using Statistical Package for Social Sciences, VER 17.0. The four factors control, heavy metals, salt and combination of heavy metal and salt were computed. Results of the Bonferoni test are expressed by alphabet letters on bar graphs.

Relative proportions of enzymes

Proportions of antioxidant enzymes were calculated by the following formula:

$$\text{Proportion of antioxidant enzymes} = \frac{\text{Specific activity of SOD in combined environment}}{\text{Specific activity of antioxidant enzyme}}$$

Results

The effects of combined heavy metal (lead chloride and lead nitrate) and salt (NaCl) stress on antioxidant enzyme activity in three week old *Vigna radiata* plants were examined. Applications of chloride and nitrate salt of lead significantly altered the superoxide dismutase (SOD) and catalase (CAT) activities in the saline (NaCl) medium (Fig. 1). SOD activities were higher in a combined lead and salt stress environment than in an environment without NaCl. Optimum activity was recorded in a plant when it was treated with a combination of 15 mg L^{-1} lead chloride and 75 mM NaCl. However, 15 mg L^{-1} lead chloride without NaCl inhibited SOD activities. Likewise, SOD activities in 15 mg L^{-1} lead chloride and nitrate together with 150 and 225 mM NaCl solution were enhanced. It was observed that SOD had a greater activity in a two stress than in a single stress environment.

Single stresses, such as lead without NaCl, showed enhancement in CAT activities, optimum activity occurring in plants treated with 15 mg L^{-1} lead nitrate (Fig 1). However, at 30 mg L^{-1} lead nitrate with or without NaCl almost complete inhibition in the CAT activity was seen. With the application of heavy metals (lead chloride and lead nitrate) in a high saline environment (150, 225 mM NaCl) fewer CAT than SOD activities were recorded. It was observed that CAT had less activity in a combined stress than in a single stress environment.

When lead chloride and nitrate were combined with a NaCl solution there was substantial improvement in ascorbate peroxidase (APX) activity compared to control (Fig 2). Maximum activities were measured when plants were treated with 15 mg L^{-1} lead nitrate together with all saline media (75, 150 and 225 mM NaCl). However, lead chloride and lead nitrate showed significant inhibition when provided without NaCl.

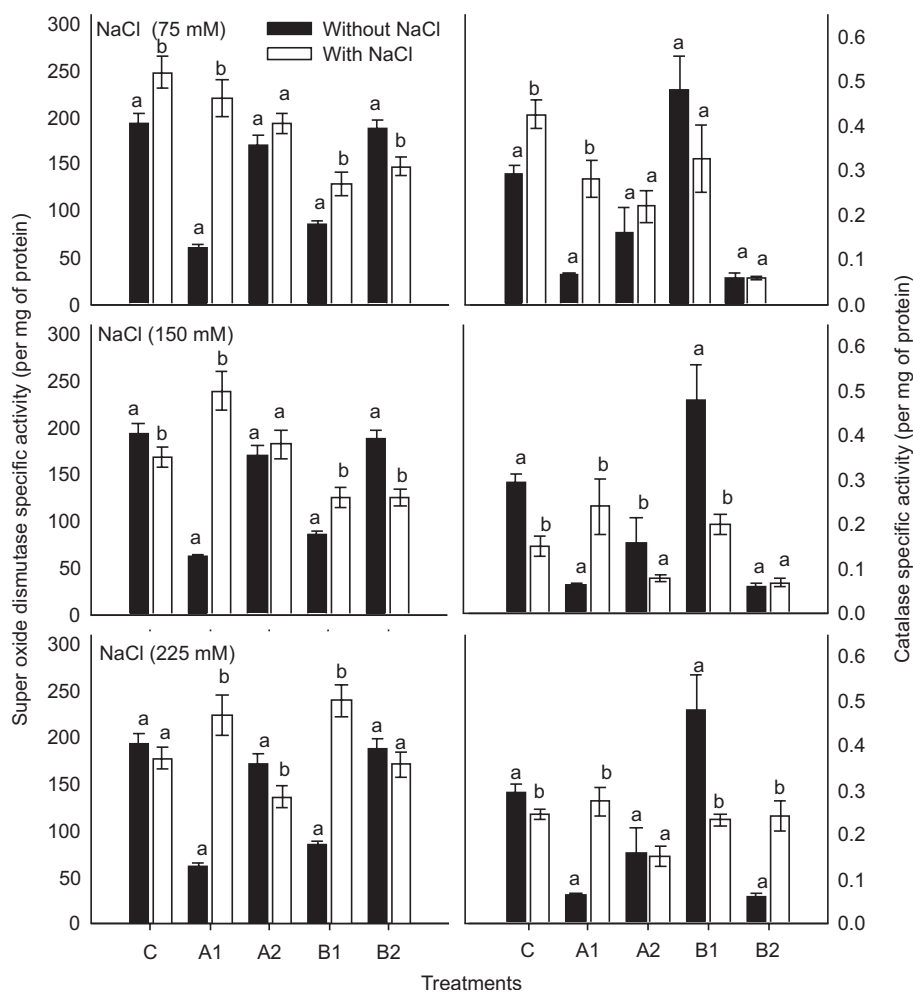


Fig. 1. Antioxidant enzyme's super oxide dismutase and catalase response in combined lead and saline environment. C – control, A- lead chloride, B – lead nitrate; B1 = 15, B2 = 30 mg L⁻¹ lead concentrations. Vertical lines on bar graphs represent mean ± SE. Similar alphabet letters show non significant difference at $p < 0.05$ from corresponding ones measured on plant treated with lead salt with and without saline environment.

Glutathione reductase (GR) activities improved in a combined stress environment, with some exceptions, showing maximum activities in a plant treated with 15 mg L⁻¹ lead chloride and 30 mg L⁻¹ lead nitrate together with all saline concentrations (Fig 2). Application of single-stress lead chloride in particular caused considerable reduction in GR activities.

Guaiacol peroxidase (GPX) activities were enhanced by the combination of lead and salt stress compared to control (Fig. 3). However, optimum activities were recorded when plants were treated with 15 mg L⁻¹ lead nitrate together with 75, 150 and 225 mM NaCl. In general, a combination of heavy metal and salt stress improved GPX activities except when 30 mg L⁻¹ lead nitrate was applied together with 150 and 225 mM NaCl.

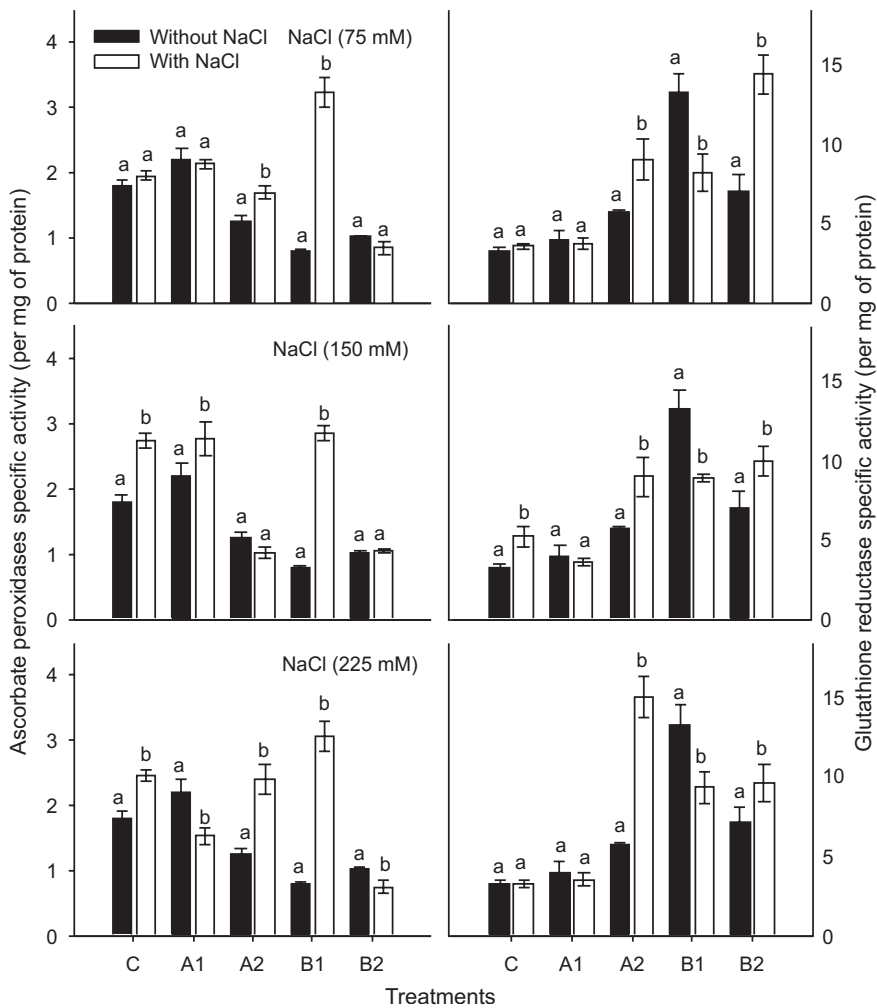


Fig. 2. Antioxidant enzyme's ascorbate peroxidase and glutathione reductase response in combined lead and saline environment. For symbol explanation see figure 1.

In order to compare the activity of antioxidant enzymes in combined stress environment, the relative enzyme activity performances in a combined stress environment were calculated. The study showed substantial activity of glutathione reductase (GR) after SOD (Fig 4). APX and GPX also had optimum activities compared to CAT. The highest SOD and GR enzyme units being found in a combined stress environment indicates a better tolerance against a combination of these stresses than found in other antioxidant enzymes.

Discussion

Antioxidant activities, in enzymes such as ascorbate peroxidase, catalase, superoxide dismutase activities are known to increase in a variety of environmental stresses like soil sa-

linity, drought, extremes of temperature and heavy metals. Physiologically these stresses cause oxidative damage to plants either directly or indirectly (COMBA et al. 1998, BAISAK et al. 1994, BECANA et al. 2000, SHAH et al. 2001, SIDDIQUI et al. 2008). In fact, oxidative stress hazards are due to the production of reactive oxygen species which include superoxide radical (O_2^+), hydroxyl radical (OH^+) and hydrogen peroxide (H_2O_2). Reactive oxygen species (ROS) products in turn cause damage to the biomolecules by peroxidation, electrophilic substitution reaction, reduction of membrane lipids, proteins, chloroplast pigments, enzymes, nucleic acids, etc. (COMBA et al. 1998, BECANA et al. 2000, MAJEED et al. 2010).

In a defense system, SOD has a critical role in plants and functionally it dismutates superoxide radicals into water and O_2 . It has been reported that SOD activities increased in rice plants growing under toxic levels of Pb and salinity (VERMA and DUBEY 2003), water stress (ZHANG and KIRKHAM 1994), Cd, Pb, Al and Cu toxicity (CHONGPRADITNUM et al. 1992, VERMA and DUBEY 2003, MALECKA et al. 2007, MAJEED et al. 2010). Increase in SOD activity in response to stress seems to be used in de-novo synthesis of the further antioxidant enzymatic protein (FADZILLA et al. 1997, VERMA and DUBEY 2003). Therefore, it is presumed that SOD activities and synthesis of other antioxidant enzymatic protein like APX, CAT, GR, GPX in a combined stress environment are linked. It may also be suggested that a combination of two or more antioxidant enzymes activities is required to provide complete tolerance (complete detoxification of ROS) to plants under combined stress in particular.

Lower catalase activity in combined lead and salt stress environments is perhaps due to combined chloride toxicities, from both lead and sodium chloride. It is presumed that a combination of two chloride stresses may retard CAT protein and thus reduce the activity. It has been observed that catalase activity under lead chloride toxicity was delayed in the removal of H_2O_2 and peroxides, which results in lipid peroxidation and thus in growth retardation. Moreover, a similar decline in catalase activity was reported under salt stress (COMBA et al. 1998), chilling (MISHRA and SINGHAL 1992), drought stress (LOZANO et al.

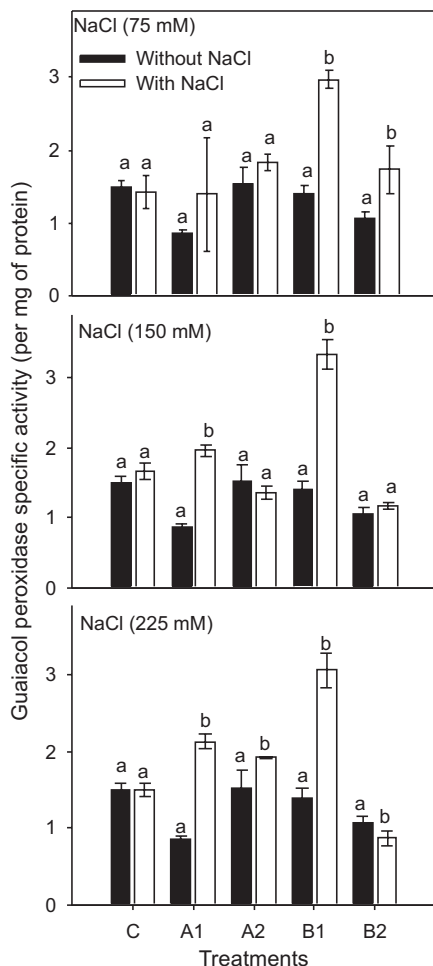


Fig. 3. Antioxidant enzyme's guaiacol peroxidase response in combined lead and saline environment. For symbol explanation see figure 1.

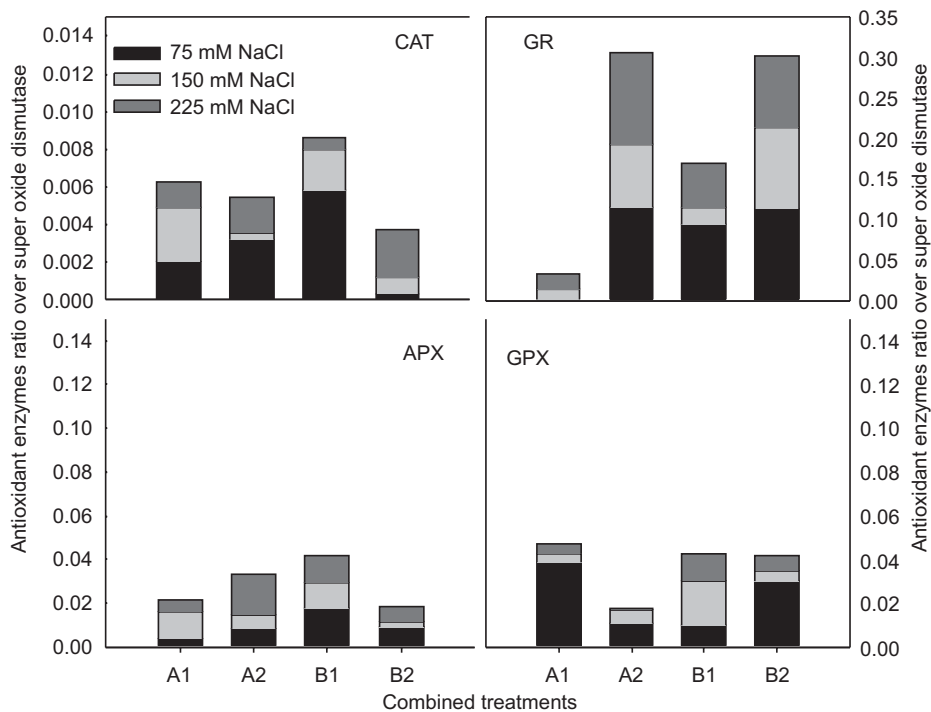


Fig. 4. Antioxidant enzymes proportion over super oxide dismutase (SOD) activity in combined lead and saline environments. APX – ascorbate peroxidase, CAT – catalase, GPX – guaiacol peroxidase, GR – glutathione reductase

1996) and hypoxia stress (USHIMARU 1992). Physiologically, reduction in catalase activity under stressful conditions has been attributed to the inactivation of enzyme protein due to ROS (USHIMARU 1992) decrease in enzyme synthesis or change in the assembly of enzyme subunits (VERMA and DUBEY 2003).

Ascorbate peroxidase (APX) guaiacol peroxidase (GPX) and glutathione reductase (GR) are crucial components after superoxide dismutase (SOD), which is required for complete neutralization of H_2O_2 (MACRAE and FERGUSON 1985, USHIMARU 1992). It is presumed that a system of antioxidant enzymes in a combined stress environment acts as a chain reaction. For instance, SOD first dismutated ROS into hydrogen peroxide which was then further converted into water and molecular oxygen by the activities of APX, GPX or GR in different proportions. It was presumed that SOD and CAT might act together to combat combined stress environment as it was observed in single stress study. However, in this study, CAT seems to be more sensitive under combined salt and lead stress. The better activity pattern of APX, GPX and GR in a combined stress environment showed a unique combination of activity patterns, not including CAT. Further, in a single stress study, APX exploits the reducing power of ascorbic acid more efficiently to get rid of potentially harmful H_2O_2 in response to abiotic stress (HERTWIG et al. 1992, JEBARA et al. 2005, SIDDIQUI et al. 2008, MAJEED et al. 2010). Likewise, glutathione reductase catalyzes the NADPH-dependent reduction of glutathione by its redox active thiol group, and glutathione is involved in the re-

dox regulation of the cell cycle (KUBO et al. 1995, WECKX and CLIJSTERS 1996, HIDEG et al. 1997, SIDDIQUI et al. 2008) and has often been considered to play a significant role in plant defense mechanisms against oxidative stress. Further the active role of GR activities has been reported by several workers in individual abiotic stress environments (KUBO et al. 1995, WECKX and CLIJSTERS 1996, HIDEG et al. 1997, SIDDIQUI et al. 2008, MAJEED et al. 2010, AHMED et al. 2010). It is presumed that GR after the SOD in a combined stress environment may be involved in regulation in a cell cycle, which may not only provide tolerance but also improve plant growth in a combined stress environment.

Therefore, it is suggested that among the five antioxidant enzymes, SOD and GR are the two prominent enzymes in combined lead and NaCl stress environment and showed more substantial activity than APX, CAT and GPX. It may also be suggested that SOD and GR enzyme proteins are less sensitive to combined lead and NaCl salt and thus provide better tolerance in a combined stress environment.

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